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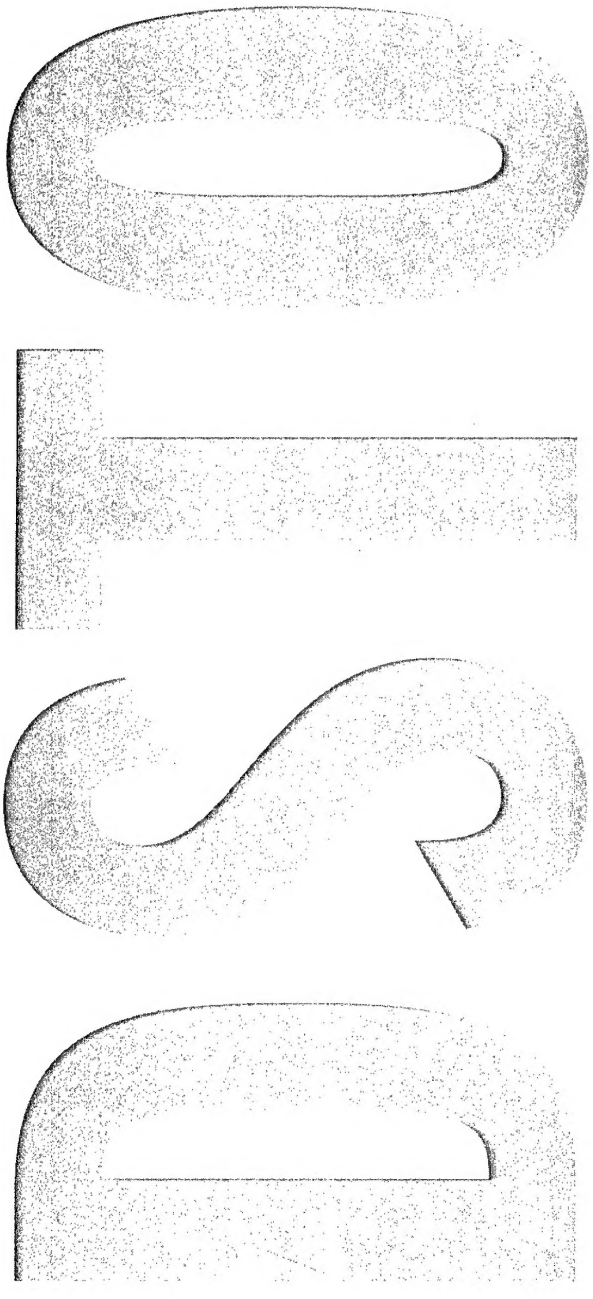

Comparison of DNA Vaccine Delivery Systems: Intramuscular Injection Versus Gene Gun Administration

Jane McAllister and David Proll

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Platforms Sciences Laboratory

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ABSTRACT

This study aimed to compare the effects of intra-muscular immunisation (IM) using needle injection with intra-dermal (ID) immunisation using a ballistic gene gun. Serum levels of antigen-specific IgG were assessed by ELISA, following immunisation with plasmid DNA encoding beta-galactosidase. It was observed that mice that were vaccinated ID displayed higher titres of antigen-specific IgG than IM vaccinated mice. This is despite the fact that only one hundredth the amount of DNA was used for ID immunisation, compared to IM immunisation In addition, the size of the gold microcarriers coupled to the DNA, that were used in the gene gun immunisations also influenced antibody production, with the larger 1.6 micron particles resulting in the highest levels of IgG. Vaccination via gene gun is therefore an efficient method of immunisation, not only for fast, safe and accurate antigen delivery, but also for activation of the humoral immune response. As a result, gene gun immunisation will be utilised in our research program aimed at developing vaccines against biowarfare agents, whenever a strong antibody response is required.

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Executive Summary

DNA vaccines have the potential to provide protection from disease caused by a range of biological warfare agents. DNA vaccination involves the direct inoculation of plasmid DNA encoding an antigen of interest into a host, where it is taken up by cells and the antigen produced, processed and transported to the cell surface, for recognition by immune cells. There are various ways to optimise the immune response, one being the type of delivery system used to administer the DNA.

The aim of this project was to compare the immunological effects of two different DNA vaccine delivery systems, in order to develop an effective strategy to administer vaccines and gain an optimal immune response as a result. Groups of mice were immunised with DNA encoding β-galactosidase either intradermally (ID) via gene gun or intramuscularly (IM) using a conventional syringe. Serum IgG antibody titres were subsequently assessed and it was found that both immunisation methods resulted in a significant humoral immune response. Gene gun immunisation however, resulted in higher antigen-specific IgG titres than IM immunisation. In addition, the size of the microcarriers on which the DNA for gene gun immunisation was coated, also influenced the level of the antibody response. Animals vaccinated with the larger diameter microcarriers generated higher IgG titres.

DNA vaccination is an advantageous method of immunisation, due to the physiochemical stability, purity and ease of production of plasmid DNA. In addition, it is possible to formulate a single vaccine that targets multiple agents. It is therefore beneficial to be able to optimise DNA vaccination strategies in order to develop safe and effective vaccines that can provide protection for ADF personnel against potential biowarfare agents.

Authors

Jane McAllister
Chemical, Biological, Radiological, Nuclear Defence Centre

Jane McAllister, joined the CBRN Defence Centre in 2003. Prior to DSTO, Jane worked at the University of Canberra, identifying potential antigens for vaccines against respiratory pathogens, and assessing their effect on the immune response. Her work in DSTO focuses on the construction and immunological analysis of DNA vaccines against biological agents.

David Proll
Chemical, Biological, Radiological, Nuclear Defence Centre

David Proll graduated from Monash University in 1993 with a B.Sc(Hons) and went on to complete his PhD in the department of Microbiology. His PhD studies focused on the replication of positive strand RNA viruses. After graduating from university he worked at the Eijkman Institute of Molecular Biology in Jakarta, Indonesia. Here, he investigated the application and development of DNA based vaccines against the parasite that causes Malaria. Upon returning to Australia in 2000, he was recruited by DSTO to initiate a research program investigating DNA vaccines for defence applications.

Contents

1. Introduction

Vaccines are effective tools for preventing infection by a large number of pathogenic organisms. To date, vaccine candidates have included recombinant protein, inactivated or live attenuated viral particles and subunit preparations (1). DNA vaccination provides an exciting new approach to vaccine development. DNA constructs encoding a desired antigen are inoculated directly into the host, where they can transfect cells and serve as templates for *in vivo* synthesis of the protein antigen, resulting in most cases in activation of both the cellular and humoral arms of the immune system.

DNA vaccines administered via intramuscular (IM) immunisation using a conventional syringe have been used in a diverse range of species (2-4). Following immunisation by this method, the DNA is either directly taken up by cells in the skeletal muscle, or travels via the blood or lymph to the draining lymph nodes or spleen where antigen presenting cells (APCs) are transfected, resulting in immune activation (5). However, intramuscular immunisation may have some limitations. Relatively large amounts of DNA are needed to induce an immune response, as the number of professional APCs residing in the muscle tissue is relatively low, and any muscle cells directly transfected lack the co-stimulatory molecules required by APCs to activate T cells (5).

An alternative to immunising with a needle is the use of a ballistic gene gun that requires the DNA to be precipitated onto gold microcarriers and uses compressed helium to propel the gold/DNA complex directly into the Langerhan's cells in the epidermis. Previous studies have indicated several advantages of intradermal (ID) immunisation using gene gun technology over intramuscular injection, in particular the efficiency of the method, as only relatively small amounts of DNA are required to elicit potent immune responses (6). This enhanced efficiency is most likely due to direct penetration of APCs by a large number of the DNA coated gold particles, thus allowing for increased antigen presentation and subsequent antigen recognition in the draining lymph nodes.

The aim of this project was to compare the level of humoral immunity obtained in mice following IM immunisation using needle injection to that seen when immunised ID using the Helios gene gun system. In addition, gold microcarriers of three different diameters were used for ID immunisation to assess whether particle size had any influence on the generation of an antigen-specific antibody response. The antigen used in this work was β-galactosidase, encoded by the Lac Z gene, which has been shown previously to elicit strong antigen-specific antibody responses with relative ease (3).

2. Materials and Methods

2.1 Plasmid purification

The plasmid pcDNA3.1/V5-His/LacZ used for vaccination was purchased from Invitrogen. The construction of the control plasmid, pcDNA3.1 start, is detailed in Proll & Gauci (9). A vial with 5 ml of LB broth (Sigma) containing 50 μg/ml ampicillin was inoculated with *E. coli* DH5α harbouring the appropriate plasmid and grown for 6hrs at 37°C with vigorous shaking. Two millilitres of this starter culture were used to inoculate 1L LB broth+ ampicillin, and incubated overnight at 37°C with vigorous shaking. Plasmid DNA was then isolated using a Qiagen megaprep kit, as per manufacturer's instructions. Following purification, DNA was precipitated by adding 0.7 volumes isopropanol and centrifuging at 14500 *g* for 20mins. The pellet was washed with 10ml cold 70% ethanol, airdried and resuspended in 3 ml H_2O. The DNA was quantitated spectrophotometrically at 260 nm.

2.2 Preparation of DNA microcarriers for gene gun immunisation

The preparation of the microcarriers was performed as described in the Biorad Helios Gene Gun System instruction manual using a microcarrier loading quantity (MLQ) of 0.5 mg/shot of gold and DNA loading ratio (DLR) of 1 μg/shot of plasmid. In brief, 100 μl of 0.05 M spermidine was added to the required amount of gold and vortexed. The designated concentration of plasmid DNA was then added, followed by the dropwise addition of 100 μl 1M $CaCl_2$ while vortexing. The mixture was allowed to precipitate at room temperature for 10min, then spun to pellet the gold, and the supernatant discarded. The pellet was then washed three times with 100% ethanol, before resuspension in ethanol containing the appropriate concentration of polyvinylpyrolidone (PVP). The gold/plasmid suspension was then coated onto tubing, and 0.5 inch cartridges prepared.

2.3 Preparation of DNA for intramuscular immunisation - endotoxin removal

Plasmid DNA was prepared for intramuscular immunisation by treatment with Triton X-114 (Sigma) to remove any residual endotoxin. Briefly, plasmid DNA extracted as described in Section 2.1, was diluted with H_2O to approximately 5.5 ml, 500 μl of Triton X-114 was added and the solution mixed thoroughly. The mixture was incubated on ice for 20 mins, followed by a 55°C incubation for 25 mins. The solution was then centrifuged at 400 *g* for 20 mins at room temperature (RT), and the upper phase removed into a new tube. This process was repeated twice before precipitating and quantitating the DNA as

described previously. The DNA was then adjusted to a concentration of 1 mg/ml in 150 mM NaCl.

2.4 Immunisation of mice

Groups of 5 female Balb/c mice (4 weeks old) were immunised on day 0, day 14 and day 28 with the relevant DNA, as summarised in Table 2 below. The groups immunised intramuscularly (IM) received 100 μg using a 21G needle, delivered by two injections each of approximately 50 μl each into the hind leg muscle. Intradermal (ID) immunisation was achieved using the Helios gene gun (Biorad), which delivered 1μg DNA at 300 psi into the shaved abdomen of each mouse. Sera were collected via intra-ocular bleed prior to initial immunisation, and subsequently on day 27. On day 41 animals were sacrificed, and blood collected via heart puncture.

Table 1: Immunisation strategy

	Plasmid	**Delivery**	**Microcarrier size**
Group 1	pcDNA3.1start	Syringe (IM)	-
Group 2	pcDNA3.1/V5-His/lacZ	Syringe (IM)	-
Group 3	pcDNA3.1start	Gene gun (ID)	1.0μ
Group 4	pcDNA3.1/V5-His/lacZ	Gene gun (ID)	0.6μ
Group 5	pcDNA3.1/V5-His/lacZ	Gene gun (ID)	1.0μ
Group 6	pcDNA3.1/V5-His/lacZ	Gene gun (ID)	1.6μ

2.5 Analysis of serum IgG by ELISA

ELISAs were performed in 96 well plates using standard methods. Briefly, the wells were coated with either 2 μg of β-galactosidase or the mouse IgG standard in 100 μl of bicarbonate buffer (0.1M Na_2CO_3, pH 9.5), and incubated overnight at 4°C. The plate was washed three times with PBS containing 0.05% Tween 20, and blocked with 3% skim milk in PBS/Tween at RT for 1 hr. The plate was washed, serum samples were diluted in 3% skim milk/PBS/Tween, and 100 μl added in triplicate to the appropriate wells. The 3% skim milk/PBS/Tween was added to standard wells. The plate was incubated at 37°C for 20 mins, followed by incubation at RT for 40 mins with agitation. After washing three times as above, 100 μl of anti-mouse IgG alkaline phosphatase conjugate (Sigma) diluted 1:2000 in PBS, was added to each well. The plate was incubated at 37°C for 30 min followed by 30 min at RT with gentle agitation. The plate was washed, and 100 μl of *p*-

nitrophenyl phosphate substrate (Sigma) added to each well. The colour was allowed to dévelop for 1 hr, before the plate was read on a microplate reader at 405 nm.

On each microtitre plate used for assaying the levels of antigen-specific IgG, a standard curve was included (see Appendix). This consisted of serial dilutions in bicarbonate coating buffer of known concentrations of mouse IgG (5μg/ml; Sigma) as listed in Appendix A1.

3. Results

To compare the level of humoral immunity obtained following ID immunisation using a gene gun, to that seen following IM immunisation via needle injection, four groups of mice were immunised with plasmid DNA containing the LacZ gene encoding β-galactosidase, a protein known to be significantly immunogenic. Three groups received 3 doses, via gene gun, of 1μg DNA coated onto gold microcarriers of diameter of 0.6 μ, 1.0 μ and 1.6 μ respectively. The fourth group received 3 doses of 100 μg DNA in saline via IM injection. Two groups of control mice received plasmid DNA consisting of the base vector that did not encode the LacZ gene product.

Sera were collected from each mouse pre-immunisation (Bleed 1), one day prior to the third immunisation (Bleed 2), and approximately 2 weeks after the third immunisation (Bleed 3). Aliquots of sera were pooled within the immunisation group, and the titres of β-galactosidase-specific IgG for each bleed measured by ELISA. Antibody concentrations were determined using a standard curve constructed from known concentrations of mouse IgG (see appendix).

As shown in Figure 1, both IM and gene gun immunisation with pcDNA3.1/V5-His/lacZ (Lac Z) resulted in significant production of anti-β-galactosidase IgG, compared to that seen in both control groups, and demonstrated a minimum twofold increase in Bleed 3 sera samples over sera from Bleed 2. However, immunisation using the gene gun, regardless of the diameter of the microcarrier on which the DNA was precipitated, demonstrated significantly higher titres of antigen-specific IgG than those receiving IM immunisation.

Within the groups immunised with the gene gun, the larger microcarriers proved more effective at eliciting an IgG response. In particular, mice immunised with the 1.6 μ gold particles displayed titres of approximately 475 μg/ml by the third bleed. This is in comparison to mice immunised with DNA complexed with 0.6μ microcarriers, which displayed lower titres of approximately 215 μg/ml. No significant amounts of antigen-specific IgG were detected in pre-immune sera from any animal.

Based on the results obtained for the pooled group sera, IgG titres of individual animals within the group were subsequently analysed (Figure 2), with similar trends observed as described above. The IgG titres rose significantly in all animals following the third vaccination. However gene gun immunisation was still superior to IM injection overall, with mice immunised with 1.6 μ microcarriers displaying antigen-specific IgG titres ranging from 407-558 μg/ml following the third immunisation, in comparison to mice immunised by IM injection that produced antibody titres of between 28-187 μg/ml.

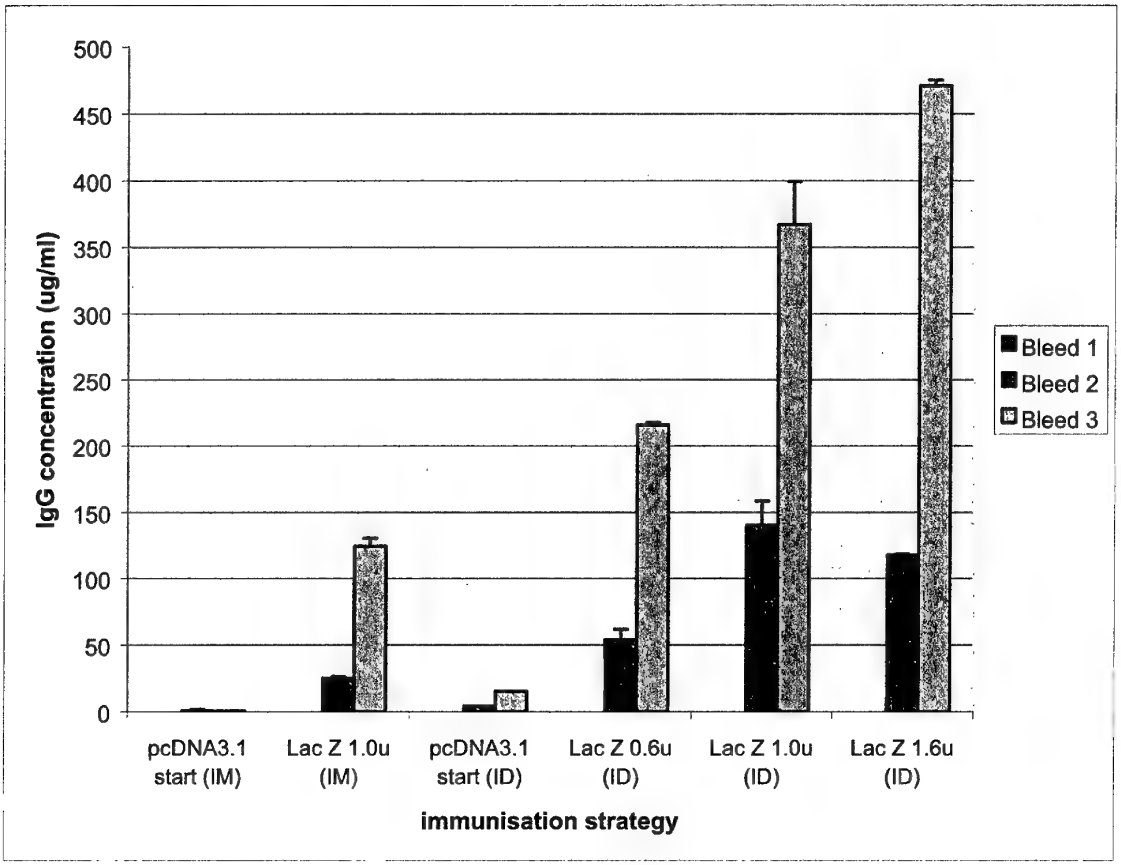

Figure 1: IgG titres in pooled sera from each immunisation groups. *Six groups of 5 female Balb/c mice were immunised with plasmid DNA containing the Lac Z gene or control plasmid (pcDNA3.1start). Animals were administered the DNA on day 0, 14 and 28, either intramuscularly (100 μg) using needle injection or via gene gun (1μg) complexed to gold microcarriers of diameter 0.6μ, 1.0μ or 1.6μ. Sera were collected from each mouse pre-immunisation (Bleed 1), one day prior to the third immunisation (Bleed 2), and approximately 2 weeks after the third immunisation (Bleed 3). Aliquots of sera were pooled within the immunisation group, and the titres of βgalactosidase-specific IgG for each bleed measured by ELISA. Values are expressed as mean IgG concentration of samples measured in triplicate ± standard error of the mean.*

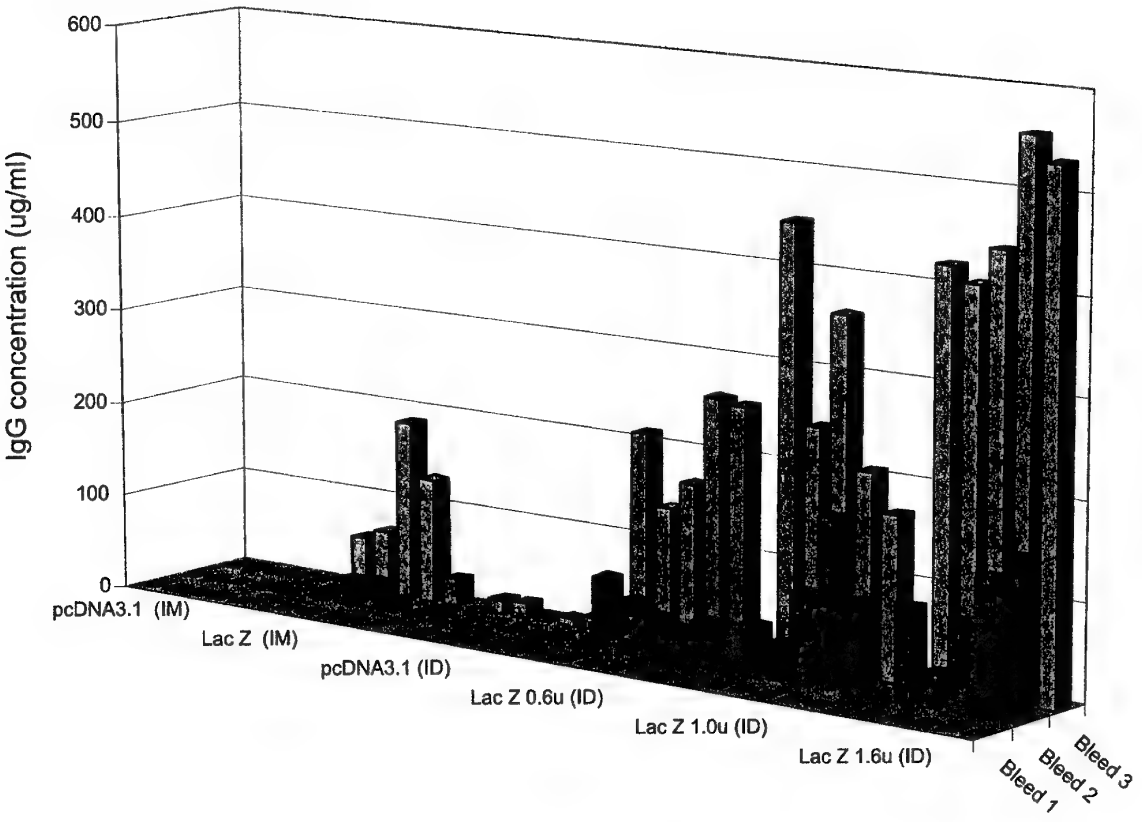

Figure 2: IgG titres in sera from individual animals following immunisation. *Six groups of 5 female Balb/c mice were immunised with plasmid DNA containing the LacZ gene or control plasmid (pCDNA3.1start). Animals were administered the DNA on day 0, 14 and 28 either intramuscularly (100 μg) using needle injection or via gene gun (1μg) complexed to gold microcarriers of diameter 0.6μ, 1.0μ or 1.6μ. Sera were collected from each mouse pre-immunisation (Bleed 1), one day prior to the third immunisation (Bleed 2), and approximately 2 weeks after the third immunisation (Bleed 3). Titres of β-galactosidase-specific IgG were measured by ELISA. Values are expressed as mean IgG concentration of samples measured in triplicate.*

4. Discussion

Delivery of plasmid DNA used in DNA vaccines can be carried out by a number of different routes, the most common being IM injection or ID delivery using a gene gun. It has been shown previously that the efficacy and nature of the immune response raised following immunisation is, in part, dependent on how the DNA is administered (7). Therefore it is important to optimise the delivery strategies of vaccines, in order to elicit an immune response that gives maximum protection.

This study has shown that immunisation of mice with a DNA vaccine encoding the Lac Z gene results in significant production of anti-β-galactosidase IgG, in comparison to mice immunised with a control plasmid that did not contain the Lac Z gene. In addition, significantly higher antibody titres were observed in groups immunised via gene gun in comparison to the group immunised IM using a needle. This is despite the fact that only 1/100 the amount of DNA is used for each vaccination with the gene gun (1μg) in comparison to needle injections (100μg). This is similar to results observed by Pertmer *et. al,* who found that when immunising IM by needle inoculation using plasmids containing human alpha-1-antitrypsin and influenza virus nucleoprotein, a 5000-fold increase in DNA was required to achieve antibody titres that were comparable to ID immunisation using a gene gun (6). The effectiveness of ballistic gene gun immunisation over IM injection can be attributed to the direct delivery of antigen into the cell. A large proportion of the plasmid DNA is essentially blasted directly into the Langerhan's cells or keratinocytes, and therefore a greater percentage of the DNA is used for antigen synthesis. In comparison, when DNA is administered via needle injection, it must enter a cell before protein synthesis can occur. The efficacy of this process is low and hence only a small proportion of the injected DNA is used for synthesis of the encoded antigen (8).

Gold particle size also proved to be an influence on the level of antigen-specific antibody production, with larger micro-carriers more effective at eliciting an IgG response. Results from previous studies have indicated that the greater the diameter of the micro-carrier, the higher the proportion of particles that enter epidermal cells (9). This subsequently results in a greater amount of DNA easily accessible for translation within the cell. In addition, the larger particle size may cause more damage to the epidermal layer. This in turn results in an increase in the level of inflammation generated (non-specific immune response), causing a greater influx of immune cells to the area of vaccination. Hence, following synthesis of the antigen encoded in the DNA vaccine there is a greater number of immune cells already present in the local area to interact with the antigen and drive the specific immune response. This ultimately results in the higher IgG titres that were observed.

As well as analysing antibody levels in the individual mice within a group (Figure 2), average IgG concentration of the individuals was also determined (data not shown) and was observed to be nearly identical to that of the pooled groups, despite some variation between animals within a group. This highlights that analysis of pooled sera can be used

to provide a quick and easy overall view of the induced antibody immune response that may be present, before going through the more time-consuming procedure of analysing individual animals.

To summarise, we have shown that the use of a gene gun to deliver DNA vaccines by ballistic propulsion is a method that results in the production of a significant immune response. It should also be noted that this method is substantially faster than using a conventional needle to deliver the DNA vaccine. Furthermore, the method would also appear to be safer from a medical health point of view as there is no need for injection and the subsequent disposal of the blood contaminated syringe. These could be important factors when completing mass vaccination of a large number of subjects or of subjects that may be carrying other potential diseases.

5. Conclusion

This study supports the findings observed in previous studies (6, 10) and concludes that ID immunisation using a ballistic gene gun is an advantageous method of vaccination over IM immunisation using a needle, for the following reasons:

- It results in a greater antigen-specific IgG immune response
- Substantially less DNA is required to achieve the greater immune response
- It is a safer, more efficient and less traumatic means of administrating the vaccine

As our research program develops DNA vaccines that can provide adequate and safe protection for ADF personnel against potential biowarfare agents, the means by which the vaccine is delivered will be an important consideration. From the results obtained in this study, gene gun immunisation will be utilised in our future research program when a strong humoral immune response is desired.

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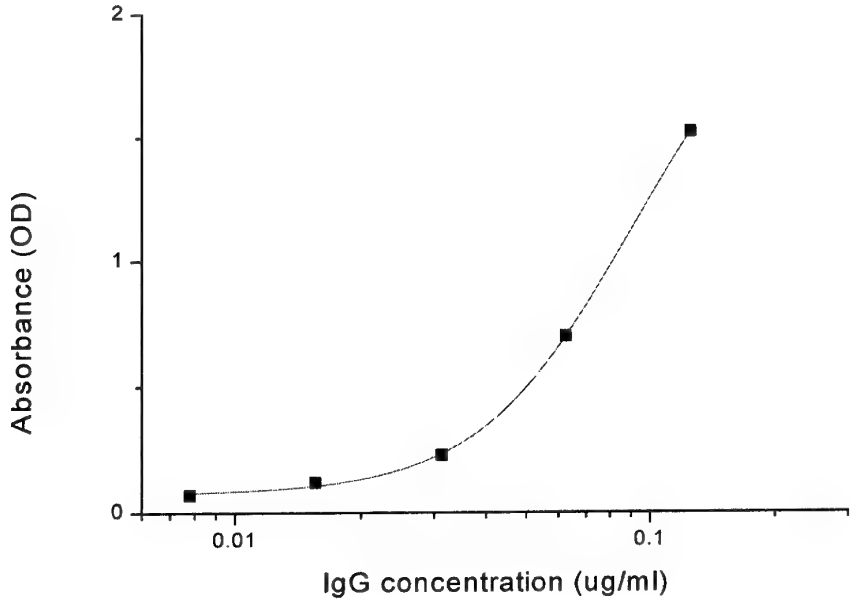
Appendix A:

A.1. Concentration of known standards used in assays measuring total antigen-specific IgG in mouse serum

	IgG (μg/ml)
S_1	0.125
S_2	0.0625
S_3	0.0313
S_4	0.0156
S_5	0.0078

A.2. Standard curve for the determination of IgG concentration in serum.



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Jane McAllister and David Proll

5. CORPORATE AUTHOR

Platforms Sciences Laboratory
506 Lorimer St
Fishermans Bend Victoria 3207 Australia

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19. ABSTRACT

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